Polypeptide Chain Structure of Rabbit Immunoglobulins. I. γG-Immunoglobulin*

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ABSTRACT: γG-immunoglobulin has been extensively reduced and alkylated and found to consist of 68% heavy chains and 32% light chains by weight. The molecular weight of the whole molecule is ~140,000, while that of the heavy chain is ~53,000 and the light chain 22,000–23,000. The extinction coefficients and partial specific volumes of the whole molecule and its chains have been measured in 5 M guanidine hydrochloride. The data are consistent with a four-chain model for γG-immunoglobulin, and the method enables one to obtain heavy chains free of light chains. It is believed that the method should prove to be valuable for studying the polypeptide chain structure of many other proteins.

The structure of γG-immunoglobulin[1] has been the subject of much interest and investigation. These studies have recently been reviewed by Cohen and Porter (1964). Edelman (1959) was the first to demonstrate the multichained nature of the γG-immunoglobulin molecule by chemical reduction. Subsequently Fleischman *et al.* (1962) developed a method for separating the two classes of polypeptide chains with good yields, and Porter (1962) proposed a four-chain model for the molecule. These partially reduced chains have been characterized as to molecular weight by Pain (1963) and as to amino acid composition by Crumpton and Wilkinson (1963). This method of preparation has been very useful since it has little effect on secondary or tertiary structure, and therefore does not destroy biological activity. Unfortunately, however, heavy chains prepared in this way have some light-chain contamination and are often partially aggregated (Porter, 1962; Nussenzweig *et al.*, 1964). To avoid aggregation Marler *et al.* (1964) measured the molecular weights of extensively reduced chains of γG-immunoglobulin in 5 M guanidine hydrochloride using a method in which separation of heavy and light chains is unnecessary. Franěk and Zikán (1964) have reduced with sulfite, fractionated with Sephadex equilibrated in 6 M urea, and found the molecule to consist of 33% light chains. Utsumi and Karush (1964) partially reduced with mercaptoethanol, fractionated with Sephadex equilibrated with detergent, and found light chains to be 28 ± 10% of the molecule.

The present paper reports in detail a method by which pure heavy and light chains can be prepared from γG-immunoglobulin and gives some characteristics of these chains. A companion paper (Lamm and Small, 1966) reports the application of this method to γM-immunoglobulin and compares some characteristics of the chains of these two classes of immunoglobulins. The principle of the method is extensive reduction in the presence of high concentrations of guanidine hydrochloride and fractionation through Sephadex G-200 in the presence of guanidine hydrochloride. In a preliminary report (Small *et al.*, 1963) we made assumptions concerning the extent of reduction as well as partial specific volumes and extinction coefficients. These parameters have now been measured. Molecular weight determinations have been repeated, this time using the high-speed sedimentation equilibrium method of Yphantis (1964).

Methods

γG-Immunoglobulin was prepared by DEAE-cellulose chromatography (Levy and Sober, 1960) of fraction II of rabbit sera obtained from Pentex, Inc., Kankakee, Ill. The purified material was shown to be free of contamination by immunoelectrophoresis with a goat antiserum to rabbit serum.

Reduction was achieved by dissolving the γG-immunoglobulin in a concentrated Tris–HCl buffer, pH 8.2, then adding β-mercaptoethanol (βME)[2], and finally adding solid guanidine hydrochloride ($\bar{\nu}$ = 0.75 ml/g; Kielley and Harrington, 1960) so that the final concentrations were: protein 2%, Tris 0.5 M, βME 0.3 M, guanidine hydrochloride 6–7 M. This mixture was incubated for 1 hr at either 37 or 25°. Addition of reagents in this order eliminated the small and

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[1] We have followed the recommendations of the World Health Organization Committee on Nomenclature for Human Immunoglobulins, whereby γM is synonomous with 19Sγ, γ_1M, or β_2M; and γG with 7Sγ, γ_2, or γ_{SS} ((1964), *Bull. World Health Organ. 30*, 447).

[2] Abbreviations used in this work: βME, β-mercaptoethanol; DTE, dithioerythritol.

variable first peak which occurs when dry protein is dissolved directly in the guanidine, Tris, βME solution (Small *et al.*, 1963).

Alkylation was achieved by mixing an equal volume of 1 M Tris, 3–4 M guanidine, and 0.36 M iodoacetamide (recrystallized two times from ethanol and petroleum ether) so that the final solution was 1% protein and 5.1–5.2 M guanidine hydrochloride. This solution was incubated at 0° for 15 min, at which time no odor of βME was present. In a few experiments, 0.1 M dithioerythritol (DTE) obtained from Cyclo Chemical Corp., Los Angeles, Calif., was used as a reducing agent in place of βME. In other experiments only an equimolar ratio of iodoacetamide was added, and then the protein was reduced a second time by the addition of βME to give a concentration of 0.3 M, and alkylation was repeated using a 10% excess of solid iodoacetamide.

Gel filtration through Sephadex G-200 in 5 M guanidine hydrochloride at room temperature has been performed in a variety of ways during the preliminary experiments as well as those reported here, and several considerations have become apparent. Polyethylene tubing must be used since the guanidine solution extracts ultraviolet-absorbing material from rubber and to some extent from tygon tubing. The flow rate decreases with time unless the effluent is run through the column in the direction opposite the force of gravity. The column used in this study was made with Sephadex G-200 which was fined 10–20 times in water and then soaked in 5 M guanidine for 3–4 days before being packed in the column. The column was run from the "bottom up," and loading was facilitated by density stabilization whereby the sample was made slightly more dense than the solution in the column and was followed with 5–10% of the column volume of a still more dense solution (5.5–5.6 M guanidine). Flow rates of 1.0–1.5 ml/cm² per hr were obtained. In all cases the guanidine solutions were made with Eastman Organic Chemicals "highest purity" guanidine hydrochloride which had been filtered through charcoal until its OD at 280 mμ was less than 0.05/cm. The pH of these solutions was 7 ± 1. Concentrations and densities of guanidine solutions were determined from refractive index measurements (Kielley and Harrington, 1960) made with an Abbe refractometer.

Column effluent was monitored at 280 mμ and the appropriate fractions were concentrated by ultrafiltration in Visking tubing (0.390-in. flat width, 0.002 in. thick), using a pressure of 1 atm for heavy chains and approximately half that for light chains (light chains may pass through dialysis tubing when 1 atm is applied). Collecting fractions by time proved most suitable.

Because of the large concentration of salt required to solubilize the fractions, extinction coefficients were determined by dialyzing protein solutions *vs.* water until all guanidine hydrochloride had been removed, then lyophilizing and allowing the protein to equilibrate with the atmosphere at room temperature. Three aliquots were then weighed and dissolved in 5 M guanidine hydrochloride, and the OD was read at 280 mμ, while six other aliquots were dried to constant weight *in vacuo* at 95° to allow estimation of the water content. Subsequently, chloride content and total ash were determined on some of the dried samples. Extinction coefficients were calculated for the dry salt-free protein.

TABLE I: Effect of Pressure on the Density of a Guanidine Hydrochloride Solution.

Pressure (bars)	ΔRefractive Index	Density	Molarity
1.0		1.1212	5.0000
142.1	0.00181	1.1238	5.1086
341.9	0.00426	1.1273	5.2556
484.8	0.00593	1.1297	5.3558

Partial specific volumes ($\bar{v}$) were determined as suggested by Casassa and Eisenberg (1960) on samples exhaustively dialyzed against 5 M guanidine hydrochloride. Concentrations were measured by absorption at 280 mμ and densities were determined in density gradient columns by the method of Linderstrøm-Lang as described in Hvidt *et al.* (1954). The density gradient columns were mixtures of *o*-dichlorobenzene and dodecane maintained at 20° with a fluctuation of less than 0.01°. It did not seem to matter whether the organic phase was saturated with 5 M guanidine hydrochloride or saturated KCl solutions. Standards consisted of KCl solutions of known density in accordance with data taken from the International Critical Tables (Washburn, 1928); weights were corrected for the buoyancy of air. Iodoacetamide, 0.04 M, was added to whole γG-immunoglobulin to inhibit disulfide exchange and the resultant increase in viscosity. A $\bar{v}$ of 0.685 was used for ribonuclease (Kielley and Harrington, 1960) and assumed to be also true for reduced and alkylated ribonuclease. Had a $\bar{v}$ of 0.707 (Reithel and Sakura, 1963) been used, ribonuclease molecular weights would have been higher (see Figures 2 and 3).

Molecular weights were determined in the Spinco Model E analytical ultracentrifuge at 20° by the high speed sedimentation equilibrium method described by Yphantis (1964), using 3-mm column heights in the six-chambered cell and interference optics. Sapphire windows were used at all speeds and protein concentrations between 0.1 and 1.2 mg/ml were employed. At low speeds the AN-J rotor was used to decrease precession, even though precession did not seem to be a problem (probably due to stabilization resulting from the redistribution of the guanidine hydrochloride). Photographic plates (Eastman Kodak IIG) were measured in a Nikon comparator which enables estimation of the tangent of the fringe as well as its *y*-coordinate at each *x*-coordinate. Weight-average molecular weights were calculated by two methods which gave very similar results, *i.e.*, least-squares fit of a ln *c vs.* r^2 plot and also extrapolation of the number-average molecular weights

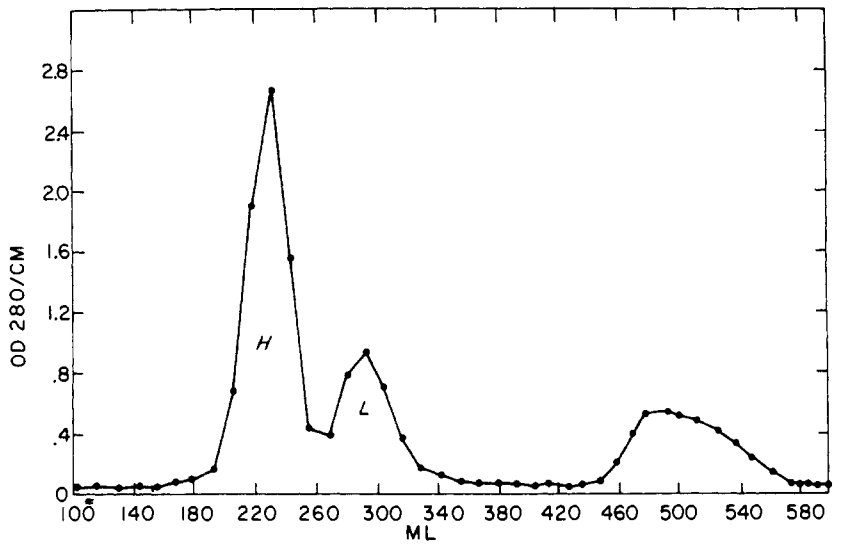

FIGURE 1: Elution diagram of γG-immunoglobulin extensively reduced and applied to a Sephadex G-200 column (120 × 2 cm) equilibrated with 5 M guanidine hydrochloride. H and L refer to heavy- and light-chain peaks, respectively, while the third peak contains small molecules (see text).

to the bottom of the cell (Yphantis, 1964). *z*-Average molecular weights were calculated from the tangent to the fringes as a function of *r* according to method II of Van Holde and Baldwin (1958). Calculations were made with the aid of a Honeywell 800 computer, and the Fortran program developed for this purpose is available (upon request to P. A. S.).

The high salt concentration in the solvent could have led to errors if, for example, the cell were improperly filled or if the solvent and solution were not thoroughly equilibrated by dialysis. To investigate this problem calculations of theoretical fringe patterns were made for unequal column heights and/or salt concentrations on the two sides of the double sector cell. They show an almost linear displacement of the fringe with respect to distance in both instances. Thus, if there is a flat depleted zone, this is good evidence for both proper filling and similar concentration of the salt in the two sectors of the cell. Furthermore, if the concentration of the salt in the solvent differs by 0.1 M from that in the solution, the fringes are so blurred as to be unmeasurable. Another source of potential inaccuracy is the change in density of the solvent due to the redistribution of guanidine in the centrifugal field and/or to the compressibility of the solvent. Calculation of the error due to the redistribution of guanidine shows this to increase the molecular weight at the bottom of the cell by less than 1% at the speeds and column heights we used. To evaluate the problem of compressibility, Waxler of the National Bureau of Standards kindly measured the refractive index of 5 M guanidine hydrochloride as a function of pressure by a method already reported (Waxler *et al.*, 1964). Since refractive index is, as a first approximation, a good measure of density, we were able to calculate the densities listed in Table I. Hence, 5 M guanidine hydrochloride is less compressible than water, and at the pressures we are concerned with (less than 50 bars) no appreciable error is introduced.

Amino acid analysis was performed according to Spackman *et al.* (1958) in a Spinco automatic amino acid analyzer. Some samples were performic acid oxidized (Schram *et al.*, 1954) before acid hydrolysis. The acid hydrolysis was performed by suspending the samples in test tubes containing 6 N HCl and placing these tubes in a desiccator. The desiccator was then evacuated and flushed with N_2 several times and finally evacuated and incubated at 110° for 24 hr.

Results

A typical elution pattern of reduced and alkylated γG-immunoglobulin from the Sephadex G-200 column is shown in Figure 1. The first peak (heavy chain) is eluted at a volume greater than the void volume and hence larger molecules, if present, would have appeared as extra peaks. The second peak consists of light chains. The third peak is eluted at a volume corresponding to the sum of the void and included volumes, and therefore represents small molecules, specifically the reducing and alkylating agents. The homogeneity of the heavy and light chain preparations was demonstrated by rechromatography, which yielded only a single peak eluting at the expected volume, and the lack of contamination of one type of chain by the other was demonstrated by disc electrophoresis experiments reported elsewhere (Small *et al.*, 1965; R. A. Reisfeld and P. A. Small, Jr., in preparation). Recoveries and percentages of the first (H) and second (L) peaks from five experiments are shown in Table II. Experiment 4 refers to material that was reduced and alkylated two times while experiment 5 deals with material that was reduced with DTE.

TABLE II: Summary of Gel Filtration Experiments.

Expt	Mg of Protein Applied to Column	Total Recovery (%)[a]		Ultraviolet (%)[b]		Mass (%)[c]	
		Ultraviolet	Mass	H	L	H	L
1	23.3	86	90	70.5	29.5	66.5	33.5
2	100	92	96	70.7	29.3	66.8	33.2
3	70	80	84	71.0	29.0	67.1	32.9
4	70	74	77	73.1	26.9	69.3	30.7
5	90	81	86	72.6	27.4	68.9	31.1
Averages		83	87	71.6 ± 1.2	28.4 ± 1.2	67.6 ± 1.3	32.4 ± 1.3

[a] Ratio of the amount of protein eluted from the columns to that originally reduced, *i.e.*, losses during reduction, alkylation, and gel filtration are included. The difference between ultraviolet and mass recovery results from the fact that the weighted sum of the extinction coefficients of the H and L chains is less than that of the whole molecule in guanidine (see Table III). [b] Ratio of the ultraviolet absorption under the specified peak (H or L) to the sum of the two peaks (H + L). [c] Ultraviolet per cent corrected for extinction coefficients (see Table III).

TABLE III: Characterization of γG-Immunoglobulin and Its Chains.

	Ash (%)	Moisture (%)	Chloride (%)	Extinction Coefficients[a,b]	Partial Specific Volume[b]
γG	2.0 ± 0.6	5.4 ± 1.1	0.3	1.36 ± 0.01	0.711 ± 0.006
H	1.2 ± 0.1	5.3 ± 1.1	0.3	1.37 ± 0.01	0.720 ± 0.004
L	0.8 ± 1.0	3.7 ± 0.6	0.2	1.14 ± 0.01	0.703 ± 0.007

[a] For 1 mg/ml solutions of dry and ash-free protein, pH ~7 at 280 mμ with a light path of 1 cm. [b] Determined in 5 M guanidine hydrochloride.

TABLE IV: Molecular Weights/1000 in 5 M Guanidine Hydrochloride.[a]

	Weight-Average[b]	z-Average[b]	Previous Method[c]	Best Value[d]
RNAase	13.6 ± 0.8[e]	12.7 ± 0.9[e]		
RNAase reduced and alkylated	13.2 ± 0.6[e]	10.0 ± 1.3[e]		
γG-Immunoglobulin				
with iodoacetamide	142 ± 5	135 ± 6		140
without iodoacetamide	172 ± 9	278 ± 53	162	
Heavy chain	56.6 ± 1.4	58.6 ± 8.8	53.5	53
Light chain	24.1 ± 0.7	28.1 ± 0.7	22	22–23

[a] Speeds used (rpm): RNAase 39,460 and 52,640; RNAase reduced and alkylated, 44,770 and 52,640; γG, 14,290 and 17,980; heavy chain, 24,630 and 35,600; light chain, 33,450 and 39,460. [b] These values represent the extrapolations of the least-square lines of Figures 2 and 3 to zero concentrations. [c] Values previously measured (Small *et al.*, 1963) with a slower speed equilibrium method and corrected for $\bar{v}$ values determined in this study. [d] See text for discussion. [e] Using $\bar{v} = 0.685$ (Kielley and Harrington, 1960).

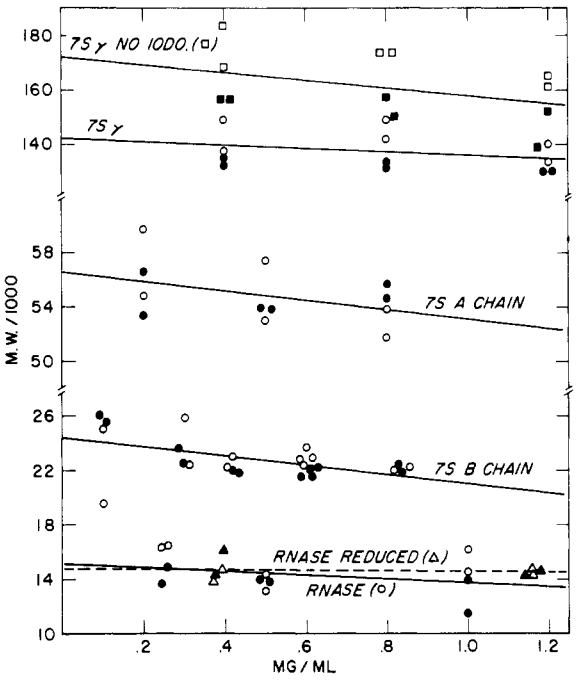

FIGURE 2: Weight-average molecular weights measured in 5 M guanidine hydrochloride. Open symbols represent determinations performed at lower speeds while closed symbols represent higher speed determinations (usually 1.5–2 times the force of gravity of the lower speed determination). The lines are least-square fits of the experimental points. 7Sγ no iodo, 7Sγ, 7S A chain, 7S B chain, RNAase, and RNAase reduced refer to γG-immunoglobulin examined in the absence of iodoacetamide, γG-immunoglobulin examined in the presence of iodoacetamide, heavy chain, light chain, ribonuclease, and ribonuclease reduced and alkylated, respectively. $\bar{\nu} = 0.707$ (Reithel and Sakura, 1963) was used to calculate the molecular weights of ribonuclease and its derivative.

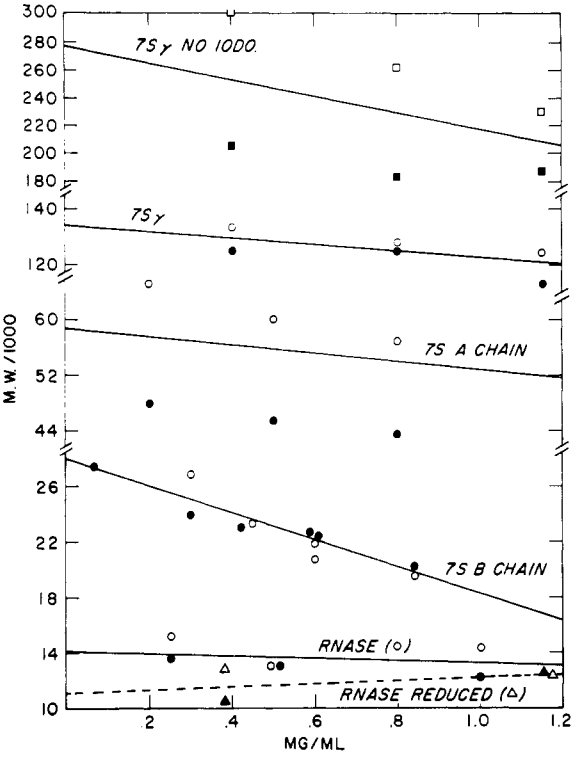

FIGURE 3: z-Average molecular weights. Otherwise the same as in Figure 2.

In Table III are listed the ash and moisture contents, the extinction coefficients, and the partial specific volumes ($\bar{\nu}$) of the whole molecule and its chains. The standard deviations of the partial specific volumes are based upon the deviations of the extinction coefficients, which are the major source of error. These values have been used for the appropriate molecular weight calculations in this paper and, since the $\bar{\nu}$ was determined as suggested by Casassa and Eisenberg (1960), the molecular weights are those of the anhydrous, salt-free molecules.

Weight- and z-average molecular weights are summarized in Table IV and are plotted as a function of concentration in Figures 2 and 3. These concentrations represent the initial concentration with which the cell was filled. After equilibrium is reached there is such a great variation in concentration across the cell that the original concentration may be less significant than is usual with such plots derived from data obtained at lower speeds. The concentration dependence, when observed, is in some instances at least in part due to fractionation in the cell of a sample contaminated with a small amount of heavier material. Thus, it seems that at higher concentrations and higher speeds, when enough material "piles up" at the bottom of the cell, the heavy contaminant is obscured there, and the resulting molecular weight is lower. This impression is supported by the observations that at higher speeds there is less concentration dependence and the molecular weights are lower for the samples in which some aggregation was suspected (*e.g.*, whole γG in guanidine without iodoacetamide). Part of the concentration dependence may also be due to the usual nonideality. Examination of the plots of the logarithm of the concentration *vs.* the radius squared yields further evidence of the degree of homogeneity or nonideality. Figure 4 shows such data for γG-immunoglobulin in guanidine in the absence of iodoacetamide. The nonlinearity of the plots is evidence of heterogeneity, which is most obvious in lower speed, lower concentration experiments (*cf.* Figures 4A and 4D). On the other hand, nonideality is best seen at higher speeds and concentrations as illustrated in Figure 8 of the following paper. Since the proteins studied in this report behaved relatively ideally and homogeneity was therefore the more relevant question, typical ln c *vs.* r^2 plots at lower speeds and concentrations are shown in Figure 5. The plots are for the most part relatively linear; however, this is not so sensitive an

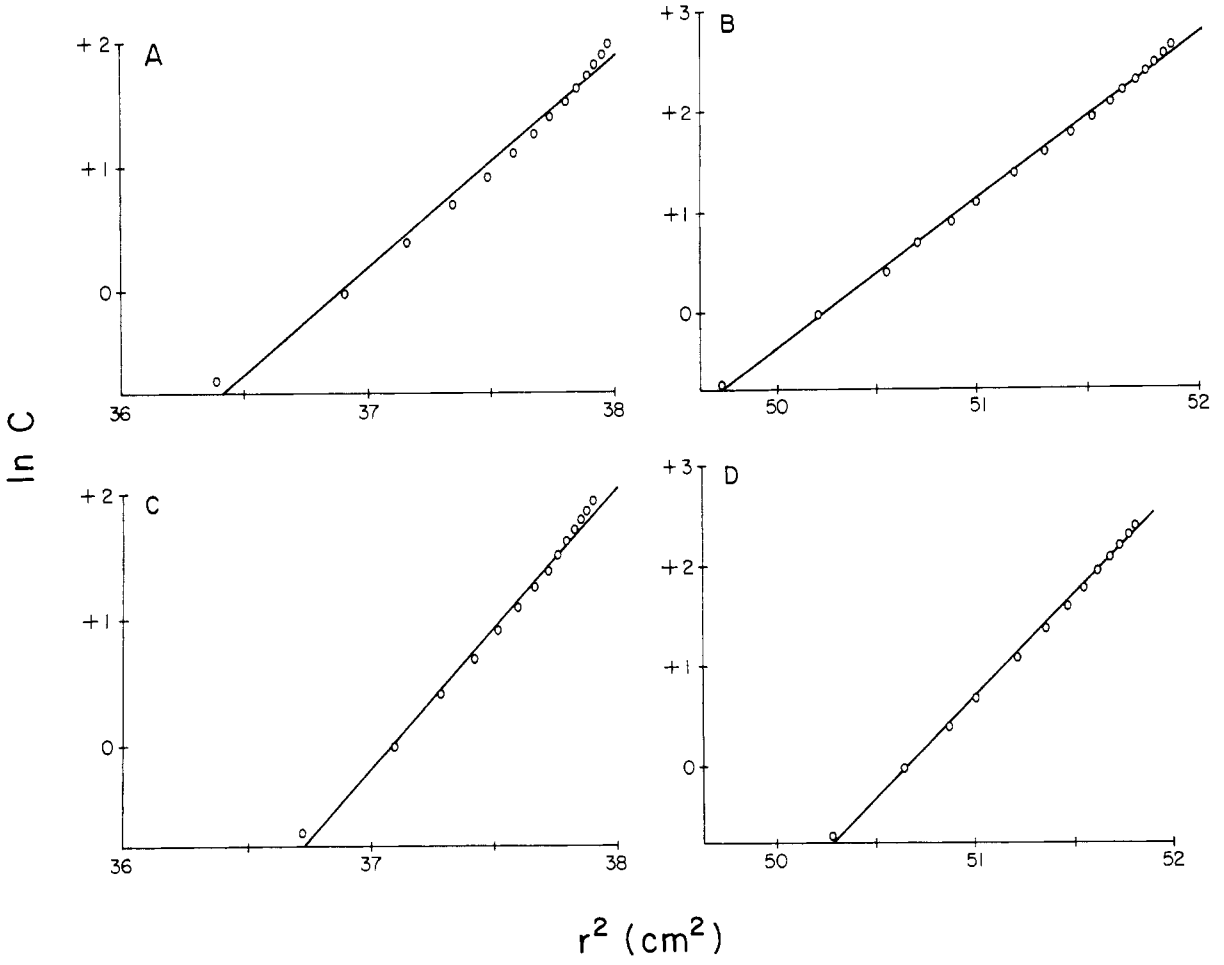

FIGURE 4: Plots of the logarithm of the concentration (in fringe numbers) *vs.* the radial distance squared for γG-immunoglobulin in 5 M guanidine hydrochloride without iodoacetamide to illustrate the effects of speed and concentration on the detection of heterogeneity. The lines are least-squares fits of the data. Concentration (mg/ml) and speed (rpm): for A, 0.4, 14,290; B, 1.2, 14,290; C, 0.4, 17,980; and D, 1.2, 17,980.

indicator of heterogeneity as a comparison of weight- and *z*-average molecular weights (Table IV). In general, weight-average molecular weights were felt to be the more accurate and *z*-average molecular weights were used principally as an indication of heterogeneity. Such considerations have allowed us to list "best" values in Table IV. Specifically, for γG-immunoglobulin there was little or no curvature of the ln *c vs.* r^2 plots (Figure 5A), the *z*-average was less than the weight-average (as in control experiments with RNAase), and molecular weights were essentially independent of speed; therefore, the "best" molecular weight was taken to be the weight-average at zero concentration. For heavy chains heterogeneity was indicated by the fact that *z*-average molecular weights were higher at the lower speed, and the ln *c vs.* r^2 plots had slight but definite upward curvatures at the bottom of the cell (Figure 5B). The "best value," therefore, is the extrapolation to zero concentration of the weight-average molecular weights at higher concentrations and higher speeds. Light chain determinations showed definite upward curvature at the bottom of the cell (Figure 5C) as well as *z*-averages higher than weight averages. This was more marked in some preparations than in others. The "best value," therefore, is again the extrapolation to zero concentration of the weight-average molecular weights at higher concentrations and at higher speeds. Although the procedure to obtain "best values" is not rigidly defined, it is of interest that similar "best values" would have been obtained by discarding readings taken near the cell bottom where deviations were obvious. For example, the molecular weight derived from the experiment depicted in Figure 5C would drop from ~26,000 to ~23,000.

Control experiments with ribonuclease are included in Figures 2, 3, and 5 and Table IV. A small molecule of this type (mol wt 13,700) necessitates centrifugation at speeds much higher than those required for the other samples, and any errors due to optical artifacts related to window strain or to differences in salt concentration

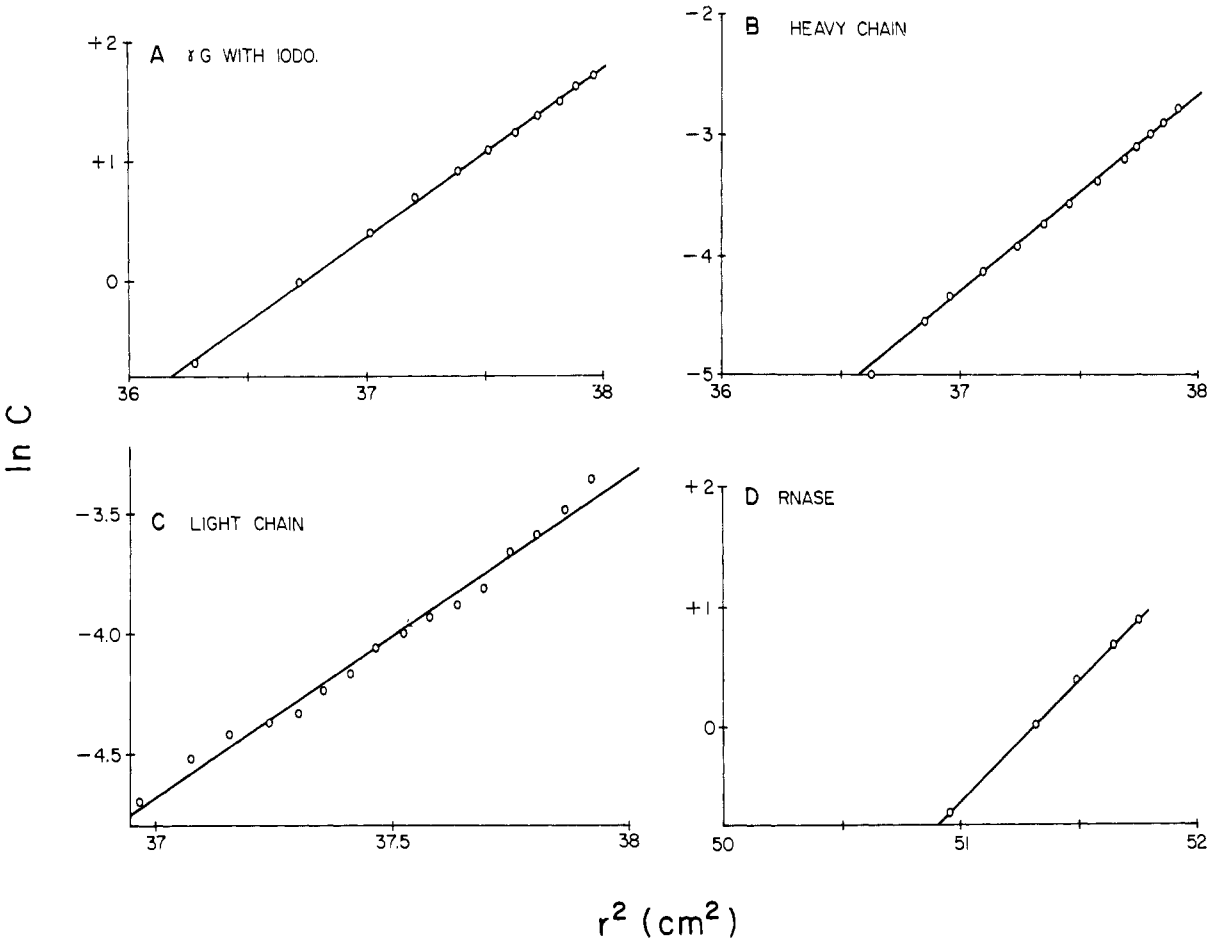

FIGURE 5: Typical ln c *vs.* r^2 plots for experiments at lower speeds and concentrations. Concentrations are in fringe numbers or in centimeters of y displacement of a single fringe from the horizontal. Solvent: 5 M guanidine hydrochloride. Lines are least-squares fits of the data. Concentration (mg/ml) and speed (rpm): for A, 0.4, 14,290; B, 0.2, 24,630; C, 0.1, 33,450; and D, 0.25, 39,460.

would be greatly accentuated. Hence, the reasonably good agreement under these extreme conditions is gratifying, and the accuracy of the method for higher molecular weight materials is probably greater.

Table V shows the cystine derivatives obtained from material that was reduced in one of three ways or performic acid oxidized before being acid hydrolyzed. The data have been normalized to that of Crumpton and Wilkinson (1963) using stable acidic and neutral amino acids (Asp, Glu, Gly, Ala, Ileu, and Leu) as the basis. Our cysteic acid value after performic acid oxidation is lower than the 36 that they found.

Discussion

In order to discuss the polypeptide chain structure of a molecule, it is first necessary to demonstrate the cleavage of all interchain bonds. Since the experiments were performed in guanidine hydrochloride, which should break noncovalent bonds, the interchain bonds of interest were the disulfide bonds. Proof of reduction of all disulfide bonds is the only sure evidence of complete cleavage of the interchain disulfide bonds, and this proof is very difficult to obtain. Table V shows that the recovery of alkylated cysteine does not equal that of cysteic acid. This difference may represent disulfide bonds that are resistant to reduction, or experimental artifacts such as differences in stability and/or rate of liberation from the protein. The figures in Table V have not been corrected for loss during hydrolysis (see discussion by Crumpton and Wilkinson, 1963). The presence of half-cystine as such after reduction with βME could result from formation of mixed disulfides, which would not be alkylated. The absence of any trace of half-cystine after reduction with DTE is consistent with this hypothesis (Cleland, 1964), and might be considered as evidence for complete reduction. However, the cysteic acid content (32.8) is greater than the alkylated cysteine (29.9). Hence the extent of reduction is still open to question. In any event the relative yields of light and heavy chain were independent of the method of reduction, and therefore if interchain disulfide bonds

TABLE V: Cystine Derivatives.[a]

	βME Reduced and Alkylated		DTE Reduced and Alkylated	Performic Acid Oxidized
	Once	Twice		
S-Carboxymethylcysteine	25.9	27.6	29.9	0
Half-cystine	4.3	0.7	0	0
Cysteic acid	0	0	0	32.8
Total	30.2	28.3	29.9	32.8

[a] Per mole of protein normalized to the data of Crumpton and Wilkinson (1963); see text.

were still present they probably represent a unique class.

The relative amounts of heavy and light chains can be obtained on a mass basis by dividing the relative per cents based on ultraviolet absorption by the extinction coefficients of the chains and normalizing to 100%. The results of such calculations are shown in the last column of Table II. The apparent agreement with our previous report (Small *et al.*, 1963) is somewhat fortuitous since those data were not corrected for the difference in the extinction coefficients of the heavy and light chains. The actual discrepancy may be due to some selective loss of heavy chain in the aggregate present in the experiments previously reported, or merely to the large scatter of values in those experiments. In any case, the yield of light chains is slightly higher than that observed by Fleischman *et al.* (1962) but comparable to that observed by Franěk and Zikán (1964).

Our value of $\bar{v}$ (0.711) for the whole molecule is lower than the 0.72 obtained by Marler *et al.* (1964) under similar conditions but using a different method. $\bar{v}$ for the whole molecule is in agreement with that calculated from the four-chain model (see below) and the partial specific volumes of the individual chains [$(0.720 \times {}^{2}/_{3}) + (0.703 \times {}^{1}/_{3}) = 0.714$].

The agreement between the molecular weights obtained in this study and those of the earlier study (when the latter are corrected for $\bar{v}$) is gratifying since two different methods of sedimentation equilibrium were used. The reason for the previously obtained high value for the molecular weight of γG-immunoglobulin is now apparent. There must have been aggregate present due to disulfide exchange, and the lower speed experiments then employed were much less sensitive for detecting heterogeneity. In the present study heterogeneity of the γG was obvious unless small concentrations (0.005 M was sufficient) of alkylating agent were added to the guanidine hydrochloride solutions. The alkylating agent presumably reacted with the small amounts of free sulfhydryl groups present in γG which were potentially capable of initiating disulfide exchange, thereby causing aggregation.

Unfortunately, the molecule is very difficult to reduce completely and there may have been some unreduced disulfide bonds in our preparations. However, the data are consistent with the four-chain model proposed by Porter (1962) wherein the approximately 140,000 molecular weight γG molecule consists of two light chains of approximately 22,000–23,000 molecular weight and two heavy chains of approximately 53,000 molecular weight.

Acknowledgments

We wish to thank the following people: Dr. Marc Lewis for helpful discussions and use of his Nikon comparator, D. Exum for performing the immunoelectrophoresis, Dr. Wm. Alford for determining Cl^- and ash content for us, and Dr. J. Potts and A. Sandoval for performing the amino acid analysis.

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Polypeptide Chain Structure of Rabbit Immunoglobulins. II. γM-Immunoglobulin*

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ABSTRACT: γM-Immunoglobulin was isolated from endotoxin-stimulated rabbits by means of ultracentrifugation, electrophoresis, gel filtration, and sedimentation through a density gradient. After reduction, alkylation, and column chromatography, all in guanidine hydrochloride, the molecule was found to contain about 78% heavy chains and 22% light chains. The hexose and hexosamine content of the heavy chain was determined.

The molecular weights of intact γM-immunoglobulin, heavy chain, and light chain were found to be 850,000–900,000, ~70,000, and 22,000–23,000 respectively. Evidence for a subunit of ~180,000 molecular weight was also obtained. These results suggest that γM-immunoglobulin is composed of five subunits, each of which contains two heavy and two light chains. Comparison of heavy chains from γM- and γG-immunoglobulin from the same rabbit showed gross differences in amino acid and carbohydrate composition, fingerprints, and molecular weight. On the other hand, the light chains had the same molecular weight and very similar fingerprints.

γM Immunoglobulins,[1] whose reported molecular weights vary from 0.75 to 1.3 × 10^6 (Kabat, 1939; Pedersen, 1945; Caputo and Appella, 1960; Kovacs and Daune, 1961; Franěk, 1962; Miller and Metzger, 1965), comprise 5–10% of the total serum immunoglobulins (Kunkel, 1960). They are not dissociated by 6.6 M urea, wide variations in temperature or pH, or ultrasonic vibrations (Putnam, 1959). Deutsch and Morton (1958) have shown that these macromolecules can be reduced by mercaptans, yielding heterogeneous subunits with sedimentation coefficients in the 6–7S range and molecular weights in the neighborhood of 180,000. These subunits contain the same percentage of carbohydrate as the parent molecules, and hence are different from γG-immunoglobulins (Kunkel, 1960).

Cohen (1963) has demonstrated that human γM-immunoglobulins are composed of two types of polypeptide chains in roughly the same proportion as the two types of chains in γG-immunoglobulin molecules. Furthermore, he and Carbonara and Heremans (1963) were able to show by immunological and electrophoretic criteria that the light chains are similar or identical in all three classes (γG, γA, and γM) of immunoglobulins, but that the heavy chains are different. Recent work on the amino acid and carbohydrate composition of the heavy and light chains of human γM- and γG-immunoglobulins has confirmed these conclusions (Chaplin *et al.*, 1965).

In this study we have isolated the heavy and light polypeptide chains of extensively reduced rabbit γM-immunoglobulin and have characterized them with respect to mass. This has enabled us to propose a tentative model for γM-immunoglobulin. In addition, we have compared the chains of γM molecules with those of γG.

Methods

Preparation and Analysis of Protein Fractions. Adult New Zealand rabbits (5–6 kg) were given ten successive daily intravenous injections of 0.25 μg of purified *S. enteritidis* endotoxin, which is almost wholly polysaccharide. They were then bled several times during the next few days by cardiac puncture. KBr was added

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[1] We have followed the recommendations of the World Health Organization Committee on Nomenclature for Human Immunoglobulins, whereby γM is synonymous with 19Sγ, γ_1M, or β_2M, and γG with 7Sγ, γ_2, or γ_{SS}((1964), *Bull. World Health Organ. 30*, 447).